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Influence of Nanoparticles on Seed Germination and Seedling Growth of *Vigna radiata L.*

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ABSTRACT

We describe a simple and cost effective method for screening the effects, of stem extract along with biogenic nanoparticles synthesized from *Cissus quadrangularis* stem extract, on *Vigna radiata* seed germination and root length. At higher concentrations (200 µl to 300 µl) of stem extract (1 - 2.5 g ml^{-1}) germination was completely inhibited. The inhibition was dose dependent. Synthesized nanoparticles were characterized by UV-visible spectroscopy, XRD and FTIR analyses. The average size of BNPs was 15.9 nm. FTIR analysis revealed resultant groups as aldehyde, ketones, lactones, alkanes, amines, etc. The BNPs (10 mg ml^{-1} and 15 mg ml^{-1} at 200 µl to 300 µl) completely inhibited seed germination.

Keywords: *Cissus quadrangularis,* seed germination, biogenic nanoparticles, *Vigna radiata,* XRD, FTIR.

INTRODUCTION

Cissus quadrangularis L is an important medicinal plant (Fam. Vitaceae) and commonly known as bone setter due to its bone fracture healing property. Three morpho-variants of *Cissus quadrangularis* with square-stemmed, round-stemmed and flat-stemmed are available. They are differentiated as variant I, II and III respectively[1]. It requires warm tropical climate and are mainly propagated by stem cuttings in months of June and July. The plant has several uses: treatment of bone fracture, diarrhea, skin disorders, irregular menstruation, piles, tumors, wounds and scurvy.

This plant has been documented in ayurveda for its medicinal uses in gout, syphilis, veneral disease, piles, leucorrhoea, dysentery and kapham[2]. The entire plant is of medicinal value, and used for the

treatment of pain and inflammation associated with hemorrhoid as well as reducing the size of hemorrhoids. The extract of *C. quadrangularis* may protect the gastric mucosa against ulceration by its antisecretory and cytoprotective property. The ethyl acetate extract and methanol extract of both fresh and dry stems exhibited antimicrobial activity against Gram-positive bacteria, stem extract has excellent osteoinductive property which makes it an ideal candidate for bone tissue engineering[3]. Thirunavukkarasu *et al.,*[4] reported the antiparasitic activities of synthesized silver nanoparticles using stem aqueous extract of *C.quadrangularis* (C*q)* against the adult *Hippobosca maculata* and the larvae of *Rhipicephalus microplus.* The biosynthesized silver nanoparticles of *C.quadrangularis* extract has shown antibacterial activity against several bacteria[5,6] .

Prema *et al.,*[7] demonstrated the potent cytotoxic activity of the combined ethanolic and ethyl acetate extracts of *Cissus quadrangularis* and *Aegle marmelos.* The cytotoxicity of samples on colon: HT-29 cells were determined by the MTT assay. The phytochemical constituents such as flavonoids and terpenoids are the major components which are responsible for the potential cytotoxic activity. Bhuvanasree *et al.,*[8] reported the rapid synthesis of gold nanoparticles (AuNP) using the aqueous extract of *Cissus quadrangularis* (CQE) by microwave irradiation. Use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes because it eliminates the elaborate process of maintaining cell cultures. Over the last decade, several novel highly active natural products have been described whose therapeutical potential for anti-cancer treatments are tested. However, due to the great number of still non-treatable kinds of cancer and their tendency to produce resistances during anticancer treatment, we are faced with a dire need to find new compounds and new lead structures for cancer chemotherapeutical purposes. Recently nano encapsulated therapeutic agents such as antineoplastic drugs have been used to selectively targeting anti tumor agents and obtaining higher drug concentration at the tumor site.

Multiple bioassay systems and experimental models are used to decipher the anti-mitotic activity. These include *in vivo* investigations based on HeP 2 and Vero cell lines and the IC-50, root tip meristem, etc. Most of these bioassays are cumbersome and time consuming. In recent years a simple *in vitro* bioassay has been successfully evaluated for the rapid and preliminary screening of plant extracts having inhibitory effects [9,10]. The efficacy of this assay system in screening for stem extract along with nanoparticles in *C. quadrangularis* is the subject matter of this paper.

MATERIALS AND METHODS

Screening effects of stem extract

Germination was evaluated in *Vigna radiata* seeds sprouted in tap water for 24 hr at room temperature. Following germination parameters: water imbibitions, % germination and seedling growth were evaluated.

Preparation of fresh stem extract

The fresh stem extract was prepared using a simple maceration process. Fifty

grams of stem was grounded separately in 10 ml of distilled water and centrifuged at 10000 rpm for 10 min and the supernatant was stored at 4°C for further use. Four stocks were prepared (1, 1.5, 2.0, 2.5 g ml^{-1}). Five working concentrations of this stock extracts were prepared as 100, 150, 200, 250, 300 μl to make up the final volume of 300 μl with distilled water and used for testing various seed germination parameters.

Biogenesis of nanoparticles

Biogenic nanoparticles (BNPs) were synthesized using 50 g of *C. quadrangularis* stem pieces and then grinded in 100 ml of triple deionized water using mortar and pestle followed by boiling for 5 min. The resulting extract was filtered through Whatmann No.1 filter paper and used for the synthesis of BNPs. The filtrate was treated with aqueous $AgNO_3$ solution (1 mM, 5 mM and 10 mM) in an Erlenmeyer flask, separately for reduction to Ag^+ ions. Different concentrations of filtrate were interacted with the $AgNO_3$ solution in different ratios (8:1, 7:1, 4:1) at room temperature (35 °C) for different time periods (1-5 hr). For further study 1mM of silver nitrate aqueous solution and stem extract (8:1) was used.

Characterization of silver nanoparticles

The synthesized BNPs were characterized by UV-Vis spectroscopy, XRD and FTIR analysis.

UV-Vis spectra analysis

The change in color was visually observed in extract incubated with $AgNO_3$ solution. Stem extract without $AgNO_3$ did not show any change in color. The color of the extract started changing from first hour of incubation and the intensity of the color increased with time. The BNPs were isolated and concentrated by repeated (4-5 times) centrifugation of the reaction mixture at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was rewashed with triple deionized water and stored at 4 °C for further use. Further the reduction of pure Ag^+ ions was monitored using UV visible spectroscopy at different wavelengths ranging from 300 to 600 nm.

Pellets were lyophilized in to powder and subjected to XRD and FTIR analysis.

XRD Analysis

The powder of BNPs was subjected to XRD analysis. Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cu Kα1 radiation was used. This involved coating of the powder on XRD grid and subjected to XRD machine. The mean size of BNPs was calculated using the Debye–Scherrer's equation

D ¼ 0:94 k=b Cos h

Where D is the average crystallite domain size perpendicular to the reflecting planes, k is the X-ray wavelength, b is the full width at half maximum (FWHM), and h is the diffraction angle.

FTIR Analysis

To identify BNPs associated biomolecules, the fourier transform infra red spectra of washed and purified BNPs

powder were recorded on SHIMADZU instrument with the sample as KBR pellet in the wave number region of 500–4,000 cm^{-1}. The FTIR spectra of plant extracts were taken before and after synthesis of BNPs and analyzed for possible functional groups for the formation of BNPs.

Stock preparation

Synthesized BNPs were used for making the stocks of 5 mg ml^{-1}, 10 mg ml^{-1}, 15 mg ml^{-1} and 20 mg ml^{-1}, respectively. Five dilutions of this stock extract were prepared using 100, 150, 200, 250 and 300 μl to make up the final volume of 300 μl with triple deionized water.

Seed germination assay

Seeds of *Vigna radiata* L used in this study were obtained from the Rajasthan State Seed Corporation, Durgapura, Jaipur. Dry seeds of equal weight were sterilized with 5% NaOCl for 2 min; followed by rinsing with sterile distilled water (4 - 5 times). The prepared dilutions were taken in well plates and sterilized dry seeds were added in each well and the plate was covered with lid, incubating at room temperature for 48 hr. Seeds were soaked in tap water in the control group for 48 hr. For morphological studies, the time of sprouting was permitted up to 72 hr and photographs were taken. The root length was measured in cm. Percentage of seed germination was evaluated in control, plant extract and anti cancerous drug (vincristine).Water was used for dilution. Water as a control and anti cancerous drug as a standard was used for study. Each experiment was performed in triplicate and mean value was computed.

RESULTS AND DISCUSSION

The response of germinating moong beans seeds is evaluated using the defined bioassay. Seed germination comprises water imbibitions, emergence of radicle followed by plumule. The quiescent embryo begins to enlarge due to water imbibitions, leading to cracking of seed coat and protrusion of radicle. The imbibitions by seeds is followed by concomitant metabolic activity of the embryo followed by active cell division leading to seedling growth.

Effect of plant extracts on seed germination

The influence of stem aqueous extract on seed germination and seedling growth was observed and compared with the synthesized BNPs as well as vincristin. The inhibition of seed germination and seedling growth was dose-dependent and helped to quantify the bioactivity of extract and BNPs. Table 1 shows the effect of different concentrations of stem extract (SE) on seed germination at low concentration were efficient to interfere with germination and radicle length**.** The SE significantly reduced % germination over control.

Among the different concentrations 200 to 300 µl (dilutions) of stocks showed complete inhibition of seed germination. 150 µl (working concentration) of 2.0-2.5 g ml^{-1} SE showed significant inhibition of seed germination (Fig. 3). With 1.0 g ml^{-1} and 1.5 g ml^{-1} of SE stock solution, 100 % seed germination was observed at 100 µl. 90 % and 60 % of seed germination was observed at 150 µl of 1.0 g ml^{-1} and 1.5 g ml^{-1} of stocks respectively. With 2.5 g ml^{-1} stock solution, 90 % seed germination was

observed at 100 µl, 150 µl of the same caused 0% germination. All of them showed inhibition to varying extent with a maximum shown by 2.5 g ml^{-1} of SE.

Seed sprouting involved active mitotic activity and the inhibition of sprouting of seeds caused by stem extract indirectly indicated anti mitotic factors. Fig. 3 and table 1 present the dose dependence of inhibition of caused by SE (1.0 – 2.5 g ml^{-1}) leading to reduced root length at higher concentrations.

Table1. % Germination and mean root length (cm), using different concentrations of fresh stem extract (SE) (g ml^{-1})

Dilutions of SE (*µ*l)	%GSE-1.0 g ml^{-1}	%GSE-1.5 g ml^{-1}	%GSE-2g ml^{-1}	%GSE-2.5 g ml^{-1}	RL(cm) SE-1.0 g ml^{-1}	RL(cm) SE-1.5 g ml^{-1}	RL(cm) SE- 2 g ml^{-1}	RL(cm) SE-2.5 g ml^{-1}
100	100%	100%	100%	90%	1.6	1.2	1.2	1
150	90%	60%	0	0	1	1	0	0
200	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0

Note- %G- per cent seed germination, control-distilled water, RL-root length

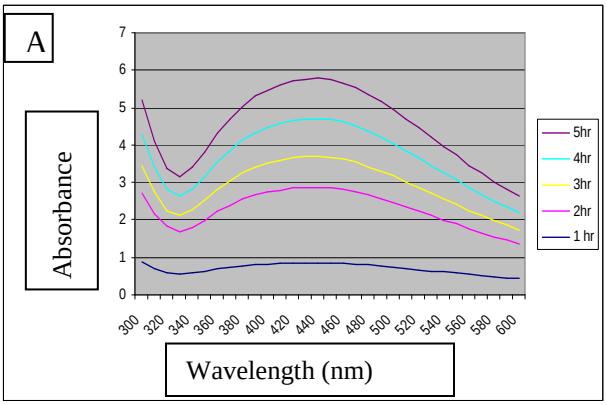


Fig 1: UV–visible spectra of the BNPs, the maximum peak was observed at 440 nm, indicating successful formation of BNPs.

Characterization of BNPs

The change in color was observed from white to brown during 5 hr of incubation, indicating the synthesis of BNPs. The production of BNPs was initiated from 50–60 min onwards and gradually increased up to 5 hr. There after 5 hr no color change occurred indicating the completion of the synthesis of NPs.

This was primarily characterized by UV–visible spectroscopy analysis. Effect of

contact time on BNPs synthesis was evaluated with UV–visible spectra and observed that with increase in reaction time the peak became sharper. Fig. 1 shows the UV–visible spectra of BNPs, measured at different time intervals from 1-5 hr. The spectrum ranged from 300–600 nm. The broadening of peaks indicated that the nanoparticles were monodispersed. The maximum peak was observed at 440 nm, which revealing successful formation of BNPs. In another study Bhuvanasree *et al.,*[8] reported the rapid synthesis of gold nanoparticles (AuNP) using the aqueous extract of *C. quadrangularis* by microwave irradiation. The UV-Visible spectroscopy of the solution obtained from reduction of hydrogen tetrachloroaurate (HAuCl4) by CQE revealed a sharp surface plasmon resonance (SPR) peak at 530 nm confirming the presence of AuNP. Marimuthu *et al.,*[11] reported absorption spectrum of *M. pudica* leaf extracts at different wavelengths ranging from 300 to 600 nm revealing a peak at 420 nm.

FTIR analysis

FTIR measurements were carried out to identify the potential functional groups of the biomolecules in the stem responsible for the reduction of the silver ions. These functional molecules are associated with BNPs. Fig. 2 (a) represents the FTIR analysis of stem reaction mixture sample. Table 2 shows data on corresponding wave number and resultant group. FTIR spectrum confirmed the presence of aromatic amines, aliphatic ether and lactones which might act as reducing agents for the synthesis of BNPs.

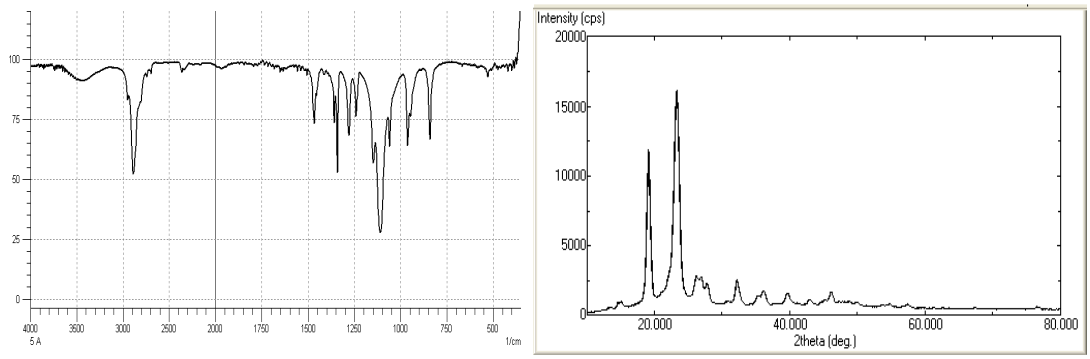
Table 2. FTIR analysis of sample 1

Corresponding wavenumber (cm^{-1})	**Type of peak**	**Resultant group**
3500	Broad-weak	Alcohol (-OH band)
2900	Medium- peak	=CH stretch, Alkanes
1480	Medium- peak	-CH bending
1300-1250	Medium peak	Aromatic aldehyde, aromatic ketones, carboxylic acid
1350	medium peak	-CN stretching (nitrile group)
1150-1100	Strong peak	-CO stretch, Acetals, aliphatic ethers, lactones
855	medium peak	-CH bending vibration aromatic ring, amines, peroxidase

XRD analysis

Crystalline size and structure of the BNPs were verified through XRD. The biosynthesized silver nanostructure by employing *C.quadrangularis* stem extract (8:1) was further demonstrated and confirmed by the characteristic peaks observed in the XRD image (Fig. 2b). The five distinct diffraction peaks of the 2 h values of 19.14^{o}, 22.02^{o}, 23.30 ^{o}, 26.16 ^{o} and 26.94^{o}, respectively indicating that the BNPs

were crystalline in nature with an average size of 15.9 nm as deduced from FWHM of peaks. Earlier Valli and Vaseeharan[12] reported biosynthesis of BNPs (50-100 nm) by *C. quadrangularis* extracts. Ag NPs were synthesized using leaf extract of *A. indica* measured 20 - 30 nm in size[13].



(a) (b)

Fig 2 Graph represents (a) FTIR Analysis (b) XRD Analysis

Seed germination as affected by synthesized BNPs

Different concentrations of synthesized BNPs, had dose dependent activity on seed germination e.g seed germination was 40% at 250 μl of 5 mg ml^{-1} (stock concentration). Seed germination was 60% and 20% with 10 mg ml^{-1} and 15 mg ml^{-1} at 150 μl , respectively. Germination was completely inhibited with 10 mg ml^{-1} and 15 mg ml^{-1}at 200-300 μl, respectively. 40% seeds germinated with 20 mg ml^{-1} at 100 μl (Fig. 4 and table 3). In control group 100% germination was observed having 3.5 cm root length.

In the present study, we report that the synthesized BNPs inhibited seed germination and seedling growth. In an earlier studies Jayaseelan *et al.*,[14] investigated the larvicidal activity against *H. maculate* of synthesized AgNPs utilizing aqueous leaf extract of *Musa paradisiaca*. Marimuthu *et al.*,[11] reported the efficacies of synthesized AgNPs using aqueous leaf extract of *Mimosa pudica* against the larvae of *R. (B.) microplus*. BNPs had more potent anti-mitotic activity than the SE, since it arrested various stages of early seed germination. With decreased dilution the inhibition of seed germination was most marked, whereas SE gave less potent results. Thirunavukkarasu *et al.*,[4] reported the antiparasitic activities of synthesized silver nanoparticles using stem aqueous extract of *C. quadrangularis*(C*q)* against the adult *Hippobosca maculate* and the larvae of *Rhipicephalus microplus.* The BNPs from extract of *Cissus quadrangularis* stem showed cytotoxicity. The cytotoxicity of the nanoparticles has been described against HeP 2 and Vero cell lines and the IC-50 value was found to be 64 μg and 90 μg, respectively [5].

In brief, reduced seed germination was associated with increase in SE concentrations as well as BNP's. At highest concentrations of the two the reduction was comparable and high. From this it is inferred that the two affected water uptake and hence osmotic potential of seeds causing reduction in turgid pressure within seed and did not permit the radicle protrusion. The retardation could be linked with a well marked reduction in mitotic index in the embryonic tissue. As discussed earlier, the phyto-constituents of the SE were analyzed using FTIR analysis which revealling the chemical composition of the plant source. This helped in identifying the compounds responsible for causing seed inhibition. The present analyses confirmed the presence of alkanes, aldehydes, ketones, phenols, aliphatic amines and lactones. It has been reported that the inhibitory effect of long chain alcohols, aldehydes, ketones increases with the enhancement in lypophilicity, as they amplify the solubility across the cell membrane. These constituents are most effective against seed germination and early seedlings growth. Lactones are also reported to inhibit germination of lettuce seeds at a concentration of 250-300 ppm[15]. This information of chemical constituents provides the reasons for the inhibition of seed germination.

Thakur *et al.,*[16] have identified different levels of chemicals in the SE of *C. quadrangularis* and these are triterpene α-amyrin acetate, aliphatic acid hexadecanoic acid and stilbene glucoside trans-resveratrol-3-O-glucoside α- amyrone, α-amyrin, β-sitosterol, kaempferol, quercetin and resveratrol. Different levels of these chemicals showed promotory attributes on the germination, and seedling growth of pearl millet at 2 and 5% concentrations of plant extracts. But at higher concentrations, the germination percentage was reduced gradually[17]. Our data correspond with their findings and those of Jadhav[18] who reported that the higher concentrations of leaf extracts of *Terminalia tomentosa*, *Sapindus emarginatus* and *Vitex negundo* inhibited the growth of field crops. But at lower concentrations, seed germination and radicle growth was promoted. The potential compounds which are able to induce inhibitory effect on seed germination are identified as phenolic acids. The reason for an inhibitory and stimulatory effect on germination percentage due to the presence of different levels of following chemicals in *C. quadrangularis* stem extracts i.e., triterpene α-amyrin acetate, aliphatic acid hexadecanoic acid and stilbene glucoside trans-resveratrol-3-o-glucosidase, etc.

Ahmad and Alamum[19] reported significant reduction in *Striga* seed germination and caused considerable seedling mortality with *C. quadrangularis* stem extract. The latter also caused 50% of *Striga* seedling mortality in about 40 days after the first seedling emergence. The results from the present study taken in conjunction with other reports point out that a combination of chemicals have significant effects on cell division, cell differentiation, ion and water uptake, water status, phytohormone metabolism, respiration, photosynthesis, enzyme function, signal transduction as well as gene expression as suggested by various studies[20]. Thus, it can be concluded that the water soluble chemicals in the SE showed potential to stimulate germination and growth at lower

concentrations and suppress the germination and growth at higher concentrations in green gram.

Table 3 shows % seed germination and mean root length, using different concentrations of BNPs

Dilutions of BNP-SE(μl)	%G BNP-SE-5 mg ml^{-1}	%G BNP-SE-10mg ml^{-1}	%G BNP-SE-15mg ml^{-1}	%G BNP-SE-20 mg ml^{-1}	RL(cm) BNP-SE-5 mg ml^{-1}	RL(cm) BNP-SE-10 mg ml^{-1}	RL(cm) BNP-SE-15 mg ml^{-1}	RL(cm) BNP-SE-20 mg ml^{-1}
100	100	80	60	40	0.56	0.52	0.46	0.36
150	80	60	20	0	0.50	0.38	0.34	0
200	60	0	0	0	0.44	0	0	0
250	40	0	0	0	0.40	0	0	0
300	40	0	0	0	0.36	0	0	0

Note: % G- per cent seed germination, RL- root length, BNP-SE- Biogenic nanoparticles-stem extract

From the above table it is clear that total inhibition of germination, at higher concentration of sample 1 did not allow any growth of the seedling. It is interesting to note that stocks of BNPs produced radicle decay.

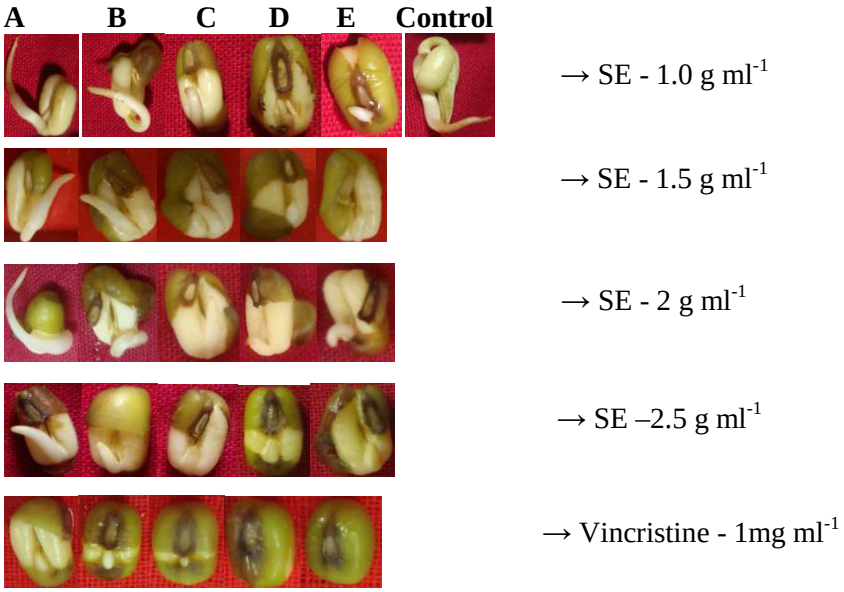


Fig 3: Seed germination, seedling emergence and growth as affected by SE – 1.0 g ml^{-1} , SE-1.5 g ml^{-1}, SE-2 g ml^{-1} and SE-2.5 g ml^{-1} using various dilutions - (a)- 100 μl, (b) 150 μl, (c) 200 μl (d) 250 μl (e) 300 μl compared with control (distilled water) and vincristine drug.

Fig 4: Seed germination, seedling emergence and growth as affected by different concentrations of BNPs– BNP-SE 5 mg ml^{-1} , BNP-SE-10 mg ml^{-1}, BNP-SE-15 mg ml^{-1} and BNP-SE-20 mg ml^{-1} using various dilutions - (a) 100 μl, (b) 150 μl, (c) 200 μl (d) 250μl (e) 300μl compared with control (distilled water).

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